# CONTROL OF SELF-FOLDING CELL-LADEN MICROPLATES BY CYTOSKELETON ALIGNMENT TO FIBRONECTIN PATTERNS

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## ABSTRACT

We propose a method to control cell driven microplate folding by fibronectin patterning. Fibronectin patterns were printed to the plates by micro contact printing. NIH/3T3 cells were seeded at a single cell level. After adhesion, cells spread and their cytoskeleton aligned to the fibronectin pattern. The microplates were detached and folded by cell traction forces of adhered cells. Our results indicate that microplate folding is dependent on the fibronectin pattern. Cell traction forces can be directed by fibronectin patterning via cytoskeleton alignment. Our approach broadens fundamental research on cell traction forces and opens further possibilities in tissue engineering.

## KEYWORDS

Fibronectin patterning, Cytoskeleton, Self-folding, Parylene, Microplate.

## INTRODUCTION

The interaction of cells with their environment influences their internal functions and cellular behaviour [1, 2]. When cells adhere to a surface, their cytoskeleton is reorganized and cells generate cell traction forces that act back on their environment [1]. Thus, the relationship between cytoskeleton orientation and cell traction forces is complex. Cell traction forces are an important parameter for tissue engineering because they affect cell shape, cell division and cell-cell contacts [1, 2, 3].

Fibronectin patterning is an established approach to support cell adhesion in microarrays. Fibronectin patterning has been applied in various forms: from mm to µm range, simple and complex shapes. Such fibronectin pattern have been demonstrated to control cytoskeleton architecture [4, 5]. In particular, line pattern of fibronectin are known to support linear alignment of actin fibers along the fibronectin lines [5].

In our previous research, cell traction forces were utilized to cause cell-laden microstructures to fold. This technique is promising for tissue engineering and bio implantable devices [6]. In former experiments, surfaces were coated uniformly with fibronectin, actin fiber orientation has not been evaluated before.

Here, we introduce self-folding microplates that are patterned with thin fibronectin lines as shown in Fig. 1. Our goal is to investigate the relationship of actin fiber orientation and microplate folding. Our preliminary results suggest that folding can be manipulated by fibronectin patterning directing cytoskeleton alignment.

## FABRICATION PROCESS

We produced pairs of 50×50 µm$^2$ parylene microplates with gaps of 3, 5 or 7 µm between the paired plates by conventional photolithography (Fig.2 A). In detail, we spin-coated 0.5% bovine gelatin diluted in Milli-Q water on a glass plate and deposited parylene C with a thickness of 3 µm (Fig.2 A1). Next, we vacuum-deposited aluminium and spin-coated photoresist S1818 (Fig.2 A2). The layout of microplates was achieved by UV exposure and photoresist etching. Then, we etched aluminium chemically and we removed the excessive parylene and gelatin by $O_2$ plasma to achieve microplates (Fig.2 A3). To ensure cell attachment to microplates only, we coated the entire surface with 2-methacryloyloxyethyl phosphoryl-choline (MPC) polymer [7], lipid that acts as a protein repellent and prevents cell adhesion (Fig.2 A4). Before experimental usage, remaining aluminium was etched with NMD-3 to lift-off MPC from the surfaces of the microplates (Fig.2 A5).

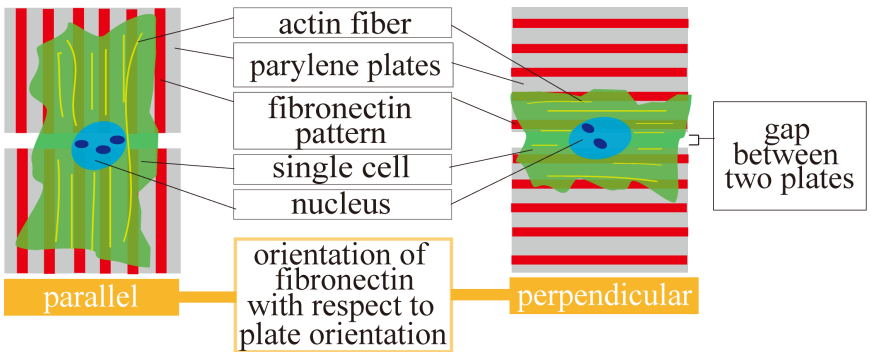


*Figure 1: Conceptional illustration of cell-laden microplate and patterned fibronectin. Fibronectin lines are printed onto microplates with a uniform thickness and a uniform pitch. Cells are seeded as single cells and after adhesion, actin fibers align parallel to the fibronectin pattern. We predict that self-folding of microplates will occur depending on the orientation of the fibronectin pattern.*



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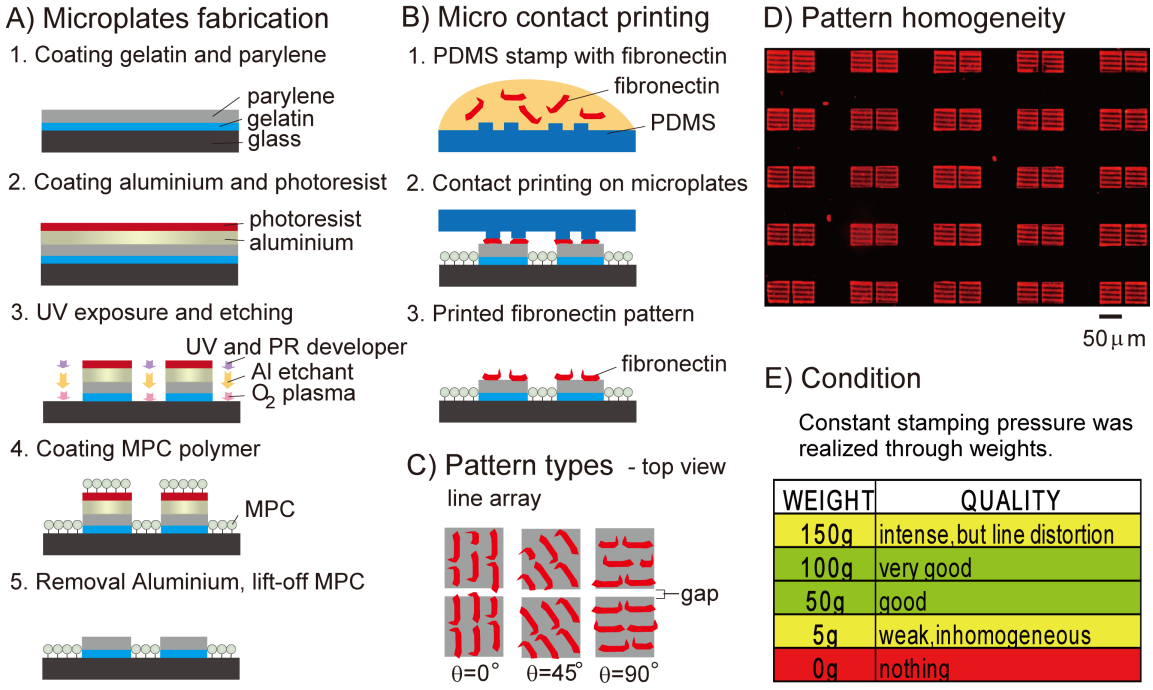


*Figure 2: A) Process flow schematic of the microplate patterning. (1)-(3) Microplates were formed by sequential etching after lithography. (4) and (5) MPC polymer [7] was coated to prevent cell adhesion. B) Microplates were patterned with fibronectin by micro contact printing with a PDMS stamp. C) Schematic illustration of pattern types. D) Fluorescence image of pairs of microplates with rhodamine tagged fibronectin line pattern. Stamps were pressed onto the sample with a weight for one hour. E) Summary of weights.*

After the MPC lift-off, we patterned fibronectin. A 1:9 mixture of rhodamine tagged fibronectin with unmodified fibronectin with a total fibronectin concentration of 50 µg/mL was printed by micro contact printing technique onto microplates (Fig.2 B, C). Line thickness of the stamps was 5 µm, line pitch differed from 5 to 20 µm. At first, the PDMS stamp was incubated in fibronectin solution for an hour at 37°C (Fig.2 B1). After an uptake of the remaining fibronectin solution, the stamp was submerged into sterile Milli-Q water twice and then gently dried under a stream of dry air. The stamp was then placed on the glass plate with its surface facing toward the microplates (Fig.2 B2). Applying pressure, the stamp was not moved for an hour at room temperature. To achieve constant and reproducible pressure, flat weights were applied. We were able to reproduce patterns with great homogeneity by using weights of 50 or 100 g (Fig.2 D, E). For control experiments, microplates were coated uniformly with fibronectin by covering microplates with fibronectin solution (50 µg/mL) for 20-30 min at room temperature.

NIH/3T3 cells were seeded onto microplates at a concentration of $5\times10^4$ cells/mL in 2 mL DMEM medium (Sigma). After 2-3 hours after seeding, the plates were washed with PBS to remove unattached cells and transferred to a new dish with fresh medium. Microscopic images were obtained at 12 and 24 hours after seeding. Staining was performed after fixation with para-formaldehyde and BSA blocking. Actin fibers were stained green with Alexa 488 phalloidin A12379 (Invitrogen) and cell nuclei were stained blue with Hoechst 33342 (Invitrogen).

## RESULTS AND DISCUSSION

In Fig. 3, we present merged fluorescence images of fixated bridging cells. Actin filaments were stained green, cell nuclei were stained blue and the underlaying 5 µm pitch fibronectin pattern is shown in red. Changing the orientation of the fibronectin lines relative to the longitudinal axis of the microplate pairs, defined as $\theta$ (Fig.3 A), affected the alignment of cells. Not only are the cell shapes aligned to the pattern, also actin fibers are oriented parallel to the fibronectin lines often. While for $\theta$=0°, cells that align to fibronectin pattern can stretch over the whole length of both microplates, cells that align to $\theta$=90° orientated fibronectin lines cannot stretch completely along the lines because the space is limited by the edges of the microplates (Fig.3 B, D).

When cells bridge between two paired miroplates, they have to cross the gap between these plates. Naturally, the greater the gap was, the more MPC coated surface cells had to bridge. For each different pattern and different gap size, we counted all single cells bridging paired microplates and normalized with all microplate pairs with attached cells at 12 hours post seeding to calculate the ratio of bridging single cells. Uniformly coated plates allow the greatest freedom for cellular movement and orientation. Uniformly coated fibronectin led to the highest ratio of bridging single cells. For a $\theta$=90° pattern, the ratio of bridging single cells was lowest. As expected, the ratio of bridging single cells is also dependent on gap size between pairs of microplates. Cells bridged less over big gaps.

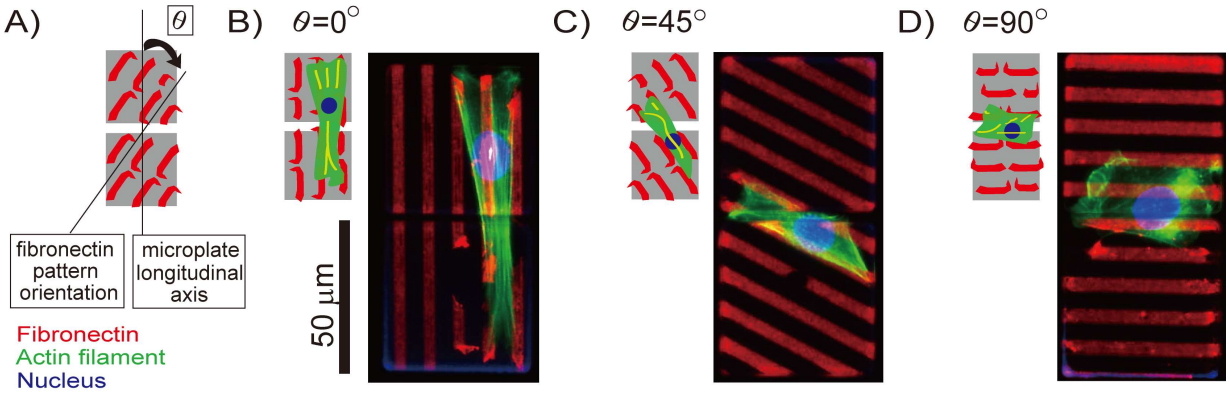


*Figure 3: NIH/3T3 cells develop different orientation and cell shapes on 50×50 µm² parylene microplates due to their alignment to different fibronectin pattern. A) The angle θ is defined between the orientation of fibronectin lines and the longitudinal axis of microplate pairs. B) - D) Fluorescence microscopy images of NIH/3T3 cells that bridge between two neighboured microplates. Actin fibers are stained green with Alexa 488 phalloidin, cell nuclei are stained blue with Hoechst 33342 and rhodamine tagged fibronectin is shown in red. Fibronectin lines are 5 µm thick and their line pitch is 5 µm. Fixation was performed at 12 hours post seeding.*

The folding of microplates pairs by cells requires a prior bridging of these cells connecting neighboured plates. Due to the underlying sacrificial layer, microplates can detach from the substrate spontaneously. When cells are attached to microplates and these microplates detach spontaneously, we often observed folding. In most cases, one microplate was detached and folded onto its neighbour plate. Thereby, cells were sandwiched between these microplates.

We counted folded microplates from bright field microscopic images at 24 hours post seeding and calculated the ratio of folded microplates. By division of that ratio and the previously obtained ratio of bridging single cells, we took into account that bridging is different for each pattern. For fibronectin lines with a pitch of 10 µm, our preliminary finding indicates that a $\theta$=0° pattern supports folding.

## CONCLUSION

We observed that actin fibers of cells often align to an underlying fibronectin pattern of thin lines. Further, our preliminary results suggest that single cells bridge microplates and cells fold microplates depending on the fibronectin line orientation.


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